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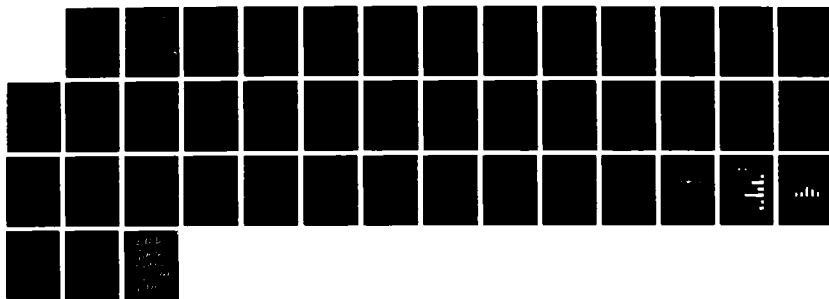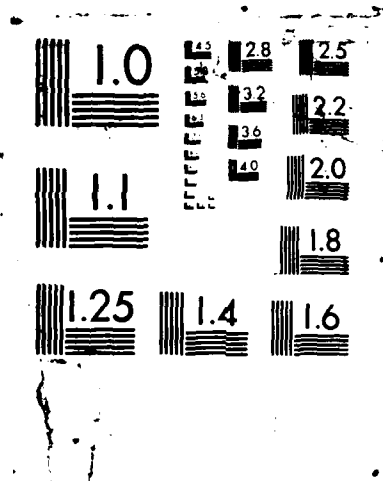30 SEP 85 DAMD17-84-C-4200   F/G 6/5   NL

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ISCHEMIC-ANOXIA OF THE CENTRAL NERVOUS SYSTEM:
IRON DEPENDEN1 OXIDATIVE INJURY DURING REPERFUSION

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ANNUAL REPORT

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SEPTEMBER 30, 1985

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This work has been undertaken to systematically characterize the contribution and time course of membrane damage by lipid peroxidation in the brain during cardiac arrest, resuscitation, and in the post-resuscitation care phase, and to develop effective interventions during resuscitation and post-resuscitation care to prevent biochemical, structural, and functional neurologic injury.

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SUMMARY

This work has been undertaken to systematically characterize the contribution and time course of membrane damage by lipid peroxidation in the brain during cardiac arrest, resuscitation, and in the post-resuscitation care phase, and to develop effective interventions during resuscitation and post-resuscitation care to prevent biochemical, structural, and functional neurologic injury.

It is now generally recognized that lipid peroxidation will not occur without catalysis by a transitional metal such as iron.[1-3] We have used a 15 minute cardiac arrest model in the dog with resuscitation by internal cardiac massage and defibrillation for our biochemical studies. During the first year we have carried out experiments indicating that tissue iron stores are delocalized from normal containment in large molecular weight chemical species to association with species of less than 20,000 dalton weight in the post-resuscitation phase.[4] The phenomenon of tissue iron delocalization specifically occurs during 2 hours of reperfusion following a 15 minute cardiac arrest; the iron delocalization is not seen after either 15 or 45 minutes of complete brain ischemia without resuscitation (Fig. I).[4] This iron delocalization is associated with the appearance of lipid peroxidation products in brain tissue.[5, 6] Studies of the fractionated lipid content of the brain cortex by chromatography 2 hours post resuscitation showed no significant differences in absolute content of individual fatty acids or of the total ratio of unsaturated to saturated fatty acids. Such differences are anticipated because lipid peroxidation occurs exclusively in unsaturated fatty acids.

Intervention in the post-resuscitation phase with the potent iron chelator deferoxamine[5, 6] in a dose of 50 mg/kg IV returns tissue concentrations of lipid peroxidation products to pre-ischemic levels after 2 hours of reperfusion (Fig. II). However, recent stochiometric estimates of the required dose of deferoxamine for chelation of all delocalized iron suggest that the dose during the first 2 hours should perhaps be as large as 150 mg/kg.

We have also studied the effect of different artificial perfusion techniques on iron delocalization during a 30 minute resuscitation period following a 15 minute cardiac arrest.[7] As indicated above 45 minutes of complete ischemia was not associated with iron delocalization. However, 30 minutes of conventional CPR did result in iron delocalization which was not seen with 30 minutes of internal cardiac massage (Fig. III). It is thus possible that the method of artificial perfusion, and the brain perfusion rates attendant on such methods may play a significant role in the initial rate of iron delocalization.

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We also carried out a preliminary prospective and blind outcome trial in which treatment with both deferoxamine and lidoflazine (a calcium antagonist) during the post resuscitation care phase were compared to standard intensive care without specific drug intervention. Five dogs were placed in each of the two study groups. After 36 hours the animals were sacrificed, immediately perfusion fixed, and the brain was evaluated by light microscopy. Neurological scoring showed no differences between the two groups at any time. Pathological findings in the standard intensive care group were characterized by marked evidence of neuronophagia in the cortex, hippocampus, and cerebellum and the presence of diffuse microhemorrhages. Animals in the treatment group were without evidence of microhemorrhages.

Both drugs were included in this preliminary outcome trial because laboratory evidence suggests a role for both iron and calcium in ischemic cell death. Preliminary evidence from Purdue[8] suggested protection from tissue calcium overloading in the early reperfusion phase by another calcium antagonist (diltiazem), and experiments in our laboratories[9] and those of Dr. Safar[10] had suggested a significant protective effect for lidoflazine. Prior to the preliminary controlled trial we had one animal achieve completely normal neurologic outcome with both drugs following a 15 minute cardiac arrest. This animal walked at 24 hours post resuscitation and has now lived in Dr. White's home for 9 months without any evidence of residual dysfunction. This recovery is without precedent to our knowledge.

Because of contradictory elements in the above data, we desired to find functional parameters of lipid membrane integrity during the reperfusion phase. We therefore undertook a study of metal ion content in the cortex over a time course during reperfusion. Atomic emission spectroscopy was used to quantitatively determine the total content of K, Na, Fe, Ca, and Mg in samples of cerebral cortex. In comparison to non-ischemic controls (n=5), there is a small increase in Ca and Na content in the cortex after 10 minutes of internal cardiac massage following a 15 minute cardiac arrest (Figs. IV and V) (n=5). All tissue ionic data is normal after 2 hours (n=5) and 4 hours (n=5) of reperfusion following resuscitation. However, after 8 hours of reperfusion (n=5), Ca and Na content have doubled, and K content has fallen to 50% normal (Fig. IV and V). There are no significant changes in total tissue content of Fe or Mg. Further discussions with the Purdue team led them to re-examine their data at 1 hour of reperfusion, and they now state that there is no significant difference between this group and non-ischemic controls in Ca content, and therefore no documented effect of calcium antagonists on the tissue content of Ca during reperfusion. Our data shows evidence of rapid recovery of ionic content control during early reperfusion from the well documented shifts occurring during ischemia.[11, 12] This normal

ionic content is maintained until a massive shift in tissue ionic content involving not only Ca, but also Na, and K, occurs between 4 and 8 hours of reperfusion. These derrangements may reflect either ionic "pump" failure or a generalized loss of permeability control reflecting physical holes produced by lipid peroxidation.

We have acquired the transmission electron microscopic capability for ultrastructural pathology and for localization of calcium.[13] We are currently repeating our studies of the molecular weight range of brain tissue iron species, the tissue concentrations of lipid peroxidation products, and the total tissue content of fatty acids (FA) and the unsaturated to saturated FA ratios after 8 hours of reperfusion. Since lipid peroxidation is known to be a fairly slow set of reactions involving bulky species,[14] it may well be that the reactions must continue for some time before substrate depletion (PUFA loss) and membrane dysfunction become evident. Electron microscopic studies and total tissue ion content studies are also being included in this detailed characterization of the point at which we now know there to be a major derrangement in membrane functions. In the next 6 months we will examine deferoxamine, oxygen radical scavengers including superoxide dismutase (SOD), catylase (CAT), and mannitol, and calcium antagonists including lidoflazine, nimodipine, and diltiazem for significant protective effects on the phenomena of iron delocalization, production of lipid peroxidation products, Na/K inversion, Ca overloading, and ultrastructural injury after eight hours of reperfusion following a 15 minute cardiac arrest. Neurologic outcome studies will then be initiated with promising agents based on this systematic and mechanistically based screening of potential therapeutic options.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication Number (NIH) 78-23, Revised 1978).

TABLE OF CONTENTS

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1. Nayini et al: Post resuscitation iron delocalization and malondialdehyde production in the brain following prolonged cardiac arrest. J Free Rad Biol Med 1:111-116, 1985.

2. Krause et al: Cardiac arrest and resuscitation: Brain iron delocalization during reperfusion. Ann Emerg Med 1985; 14:1037-1043.

3. Komara et al: Brain iron delocalization and lipid peroxidation following cardiac arrest. Ann Emerg Med 1986; 15:384-389.

4. Joyce et al: Brain iron delocalization during various methods of artificial perfusion following cardiac arrest. Resuscitation in press 1985.

5. Kumar et al: Ionic and ultrastructural changes in global ischemic dog brain. Submitted to International Academy of Pathology.

BODY OF REPORT

The Problem:

Closed chest cardiac massage has been the fundamental therapeutic maneuver in resuscitation efforts following cardiac arrest since its introduction in the early 1960s.[15] It was reasoned that provision of some artificially circulated oxygenated blood to vital organs would deter, postpone, or otherwise minimize ischemic injury until normal hemodynamics could be restored. This hope has not been fulfilled by clinical and experimental experience.[16-28]

Neurologic injury has been the major limiting element in resuscitation.[17-23, 28] Although the brain is exquisitely sensitive to invivo ischemic insult, it became apparent from the invitro work done by Ames,[29, 30] and by Hossman[31] that the neurons have substantial intrinsic resistance to complete ischemic-anoxia. The evidence shows that neurons can tolerate between 20 and 60 minutes of complete ischemic-anoxia without irreversible injury, as evaluated by recovery of ATP levels, protein synthetic capability, and action potential generation following these severe insults.

This evidence for intrinsic neuronal resistance to ischemic-anoxia led to a search for chemical and physiologic triggers resulting in neurological deficits following much shorter invivo insults. This search has produced four concepts which are the focus of both mechanistic and intervention experiments today.

1. Equilibration of Ca^{2+} between intra and extracellular fluid in the brain overloads neurons with Ca^{2+} during ischemic-anoxia and may be the initiating step leading to neuronal death.[11, 12, 32]
2. Progressive post-resuscitation hypoperfusion of the brain follows prolonged ischemic-anoxia and is related neither to changes in intracranial pressure (ICP) nor to any apparent intravascular clotting reactions.[33-37] Brain perfusion falls to about 20% normal within 90 minutes post reperfusion and remains at this low level for at least 18 hours.[38]
3. Although energy depletion is complete after 4 minutes of complete ischemic-anoxia,[39] there is continuing production of arachidonic acid by hydrolysis of membrane phospholipids in ischemic neurons for 2 to 3 hours.[40] This finding is the best characterized biochemical reaction that occurs continuously during the transition from reversible to irreversible injury during ischemia.
4. During the reperfusion phase, oxygen radicals may be produced.[41]

Background:

Injury Mechanisms: Calcium Ion Homeostasis and Shifts with Ischemia

Intracellular calcium ion concentration is normally maintained near 0.1 uM, which is 10,000 fold less than the extracellular Ca^{2+} concentration.[42] This large concentration gradient is maintained by at least four energy dependent pumping systems as follows:[43]

1. A unidirectional pump in the cell membrane which moves Ca^{2+} out of the cell and is directly ATP dependent.
2. A Na^+ -Ca^{2+} exchange pump in the cell membrane.
3. A transport system in the mitochondrial membrane which uses the membrane potential established by substrate oxidation and proton pumping to sequester Ca^{2+} in the mitochondrial matrix.
4. A transport system in endoplasmic reticulum which uses ATP to establish a H^+ gradient across its membrane, and then uses that gradient to provide the energy for Ca^{2+} uptake.

Complete ischemic-anoxia in every major organ system causes rapid decay of the normal Ca^{2+} gradient across the cell membrane. In the brain, equilibration of the extracellular and neuronal Ca^{2+} concentrations occurs during the first 5 minutes of complete ischemic-anoxia.[11, 12] Similarly, rapid shifts of Ca^{2+} into arterial walls occur and are accompanied by vascular spasm.[44]

Recognition of this massive early shift of Ca^{2+} was quickly followed by interest in the hypothesis that neuronal Ca^{2+} overloading was the primary process initiating chemical cascades leading to cell death.[32, 39] Thus, there has been intense interest in the therapeutic hypothesis that calcium antagonists may have a major role in the clinical amelioration of post ischemic brain injury.

Several experiments have now been done which support the hypothesis of post-ischemic protection by Ca^{2+} antagonists. Hoffmeister and Kazda[45] reported protection of both post-ischemic perfusion and neurologic function in cats in experiments in which the animals were pretreated with nimodipine prior to complete ischemic-anoxic insults. Steen et al.[46] have demonstrated improved neurologic recovery and protection from brain hypoperfusion following 17 minutes of brain ischemic-anoxia when monkeys were treated with nimodipine post resuscitation. '

We also found evidence of amelioration of neurologic deficits in the first 12 hours following a 15 minute cardiac arrest when lidoflazine was administered by IV drip during the

first 10 minutes post resuscitation in a prospective and blind study.[9] It is important that internal massage was used to provide perfusion during resuscitation in this and all our experiments which require definitive resuscitation. We chose this technique both because it consistently gave successful resuscitations following this period of arrest and because it provided normal cerebral cortical blood flow during resuscitation.[16, 33, 47] We thus avoided the additional low brain perfusion insult (3-10% normal) which occurs during conventional CPR.[25-28, 33] Waquier et al.[48] have also reported functional neurologic protection by flunarizine, in post insult treatment experiments in rats. Schwartz[49] has reported results of a study using verapamil and magnesium on cardiac arrest patients in the immediate post resuscitation phase; 35% of the treated patients were neurologically intact 3 months post arrest versus 0% of patients not treated with Ca^{2+} antagonists.

Injury Mechanisms: Delayed Neurologic Injury Following Cardiac Resuscitation

There are several pieces of evidence which suggest a phenomenon of delayed neurologic injury following brain ischemic-anoxia. Dr. Karl Arfors in Uppsala, Sweden has now done 15 minute global brain ischemia studies in 250 rats, one-half of which have been treated with lidoflazine and mannitol during reperfusion.(unpublished data) While untreated controls demonstrate continuous maximum neurologic deficits post insult, the treated animals appear quite normal between 12 and 18 hours post insult. However, between 18 and 24 hours post reperfusion, they all develop grand mal seizures and major neurologic deficits and then go on to die.

In our study of lidoflazine treatment of dogs post 15 minute cardiac arrest[9] we stopped neurologic scoring at 12 hours because by 14 hours all of the untreated controls had died in spite of maximum intensive care. We were able to keep treated animals alive indefinitely. However, at about 36 hours post resuscitation, these animals developed seizure activity and opstotonus which did not resolve during further observation. Similar observations have been made in Dr. Safar's laboratory.

Thus, there appeared to be a significant phenomenon of delayed neurologic decay in some experimental animals. This phenomenon is difficult to explain and suggests an ongoing mechanism of cellular injury. Lipid peroxidative reactions can continue for days once they are initiated,[14] and this type mechanism or direct injury to the DNA code by hydroxyl radical (OH*)[50] could explain the delayed functional injury. Thus, the resolution of the uncertainties which surround the proposed radical injury mechanisms assume it is urgent.

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Injury Mechanisms: Oxygen Free Radicals and Post-Ischemic Cell
Injury

Free radicals are very reactive chemical species and have
been shown to attack membranes, enzymes, and DNA, and to
seriously damage mitochondria.[50] The biological production of
the superoxide ion (O_2^-) was first identified in the reaction of
xanthine-oxidase on its substrate xanthine.[51] This classical
invitro model has recently become more interesting with the
demonstration that normal xanthine dehydrogenase is converted to
the oxidase form during the first 2 minutes of ischemia;
moreover, allopurinol, a specific inhibitor of xanthine oxidase,
has demonstrated invivo tissue protection during post ischemic
reperfusion of the bowel.[52]

Three theories might have accounted for abnormal production
of oxygen radicals during reperfusion after ischemia.

1. The conversion of xanthine dehydrogenase to the O_2^--
    producing xanthine oxidase during ischemia,[52] plus the
    accumulation of xanthine[53, 54] during anoxia-induced ATP
    depletion, could result in a large increase in the
    production of O_2^- during reperfusion.
2. The second theory is based on reducing equivalents
    accumulating during anoxia due to the lack of mitochondrial
    oxidative phosphorylation.[55] This, coupled with the
    degradation of AMP to xanthine resulting in less ADP being
    available for oxidative phosphorylation, may result in
    excessive mitochondrial reduction of oxygen to O_2^- upon
    reperfusion.
3. The third theory was based on increased levels of "free
    iron"* occurring intracellularly during or following
    ischemia.[5] This could be caused during ischemia by Ca^{2+}
    mediated mitochondrial injury[56, 57] with liberation of a
    labile pool of mitochondrial iron,[58] or by release of
    ferrous iron from ferritin by reducing equivalents
    accumulated during ischemia.[55, 59] Iron could also be
    released during reperfusion by the liberation of ferrous
    iron from ferritin in association with xanthine oxidase
    activity during reperfusion,[60, 61] The chelation of this
    Fe^{2+} by ADP accumulated during ischemia would enable
    initiation of radical lipid peroxidation via auto-oxidation
    complex as described by Aust.[4, 62]
    $$ADP-Fe^{2+} + O_2 \qquad ADP-Fe^{3+}O_2-$$

------

*   It may be that there is no such form of "free" iron,
especially since ferric iron is insoluble. Most iron is bound
up in hemoproteins, enzymes, transferrin, or ferritin where it
is normally unavailable for peroxidative reactions. The release
of iron from these chemical species would result in what we will
call "free iron." ADP chelated iron, in this sense, can be
regarded as "free" iron because it is not inert, as it is in
ferritin for example.

There are at least three biological systems which have been identified which serve to neutralize free radicals occurring during normal metabolism. Laboratory attempts to confirm the role of the free radicals in post ischemic brain injury had used these systems to try to assay for evidence of free radical activity. The three neutralizing systems include:[50]

1. Superoxide-dismutase (SOD) and catalase.
2. Glutathione-peroxidase.
3. Radical scavenging compounds, such as ascorbic acid and vitamin E.

The evidence for the role of free radicals in post ischemic brain injury was inconclusive. Demopolous et al.[41] found that the level of ascorbic acid was very low in the post-ischemic brain, and interpreted this to mean that the ascorbic acid had been consumed by free radicals. Cooper et al.,[63] however, were unable to confirm that ascorbic acid concentrations were abnormally low in the post-ischemic brain. Siesjo et al.[64] studied the ratio of reduced glutathione to the oxidized glutathione dimer which is produced by glutathione peroxidase. They were unable to demonstrate a change in this ratio in the post ischemic brain, and they suggested that this evidence argued against a major role for the free radicals in the post ischemic brain. This experiment, however, must now be viewed in light of the absence of glutathione peroxidase in the cortex, cerebellum and hippocampus.[65] Moreover, Watson et al. have demonstrated massive lipid peroxidation in brain homogenates without glutathione ratio changes.[66]

There was more direct evidence for the importance of free radicals in the destructive process following blunt injury of the spinal cord. Malondialdehyde (MDA) is an end product of lipid metabolism by free radical reactions,[67, 68] and Anderson and Means[69] provided direct evidence that it is elevated in the post traumatic spinal cord. Moreover, pretreatment of the experimental cats with anti-oxidants vitamin E and selenium produced cord protection as assayed by both structural examination and MDA assays. It is important that these same investigators were able to induce structural and chemical changes, similar to those seen with blunt trauma, by injecting small amounts of $FeCl_2$ into the spinal cord. Injection of saline did not produce similar injury.

Injury Mechanisms: Evidence for the Involvement of Iron in Initiation of Free Radical Species

The involvement of iron containing chemical species in the initiation of lipid peroxidation and in enzymatic systems which may produce O_2^- has received considerable attention. Several enzymatic systems which may produce O_2^- have iron complexes in the active sites (xanthine oxidase, cytochrome oxidase, lipoxygenase). Moreover, ferrous chelate complexes (EDTA-Fe^{2+},

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ADP-Fe^{2+}, and AMP-Fe^{2+}) have now been clearly shown to initiate lipid peroxidation.[4, 62, 70] The rate of radical oxidation in tissue homogenates is largely determined by their free iron content.[71]

The reaction of polyunsaturated fatty acids, such as arachidonic acid, with oxygen is thermodynamically unfavorable under biological conditions. Likewise, lipid hydroperoxides are relatively stable. However, in the presence of chelated transition metals (iron, copper, etc.) the peroxidation of unsaturated fatty acids proceeds and lipid hydroperoxides are very unstable. Also, superoxide and hydrogen peroxide are incapable of initiating lipid peroxidation in the absence of metals.[4, 72, 73]

There appear to be two mechanisms by which lipid peroxidation can be initiated invitro and the mechanism involved may depend on the iron chelator. The first mechanism for the initiation of lipid peroxidation is via the hydroxyl radical, which is produced by an iron-catalyzed Haber-Weiss reaction:

$$O_2^- + Fe^{+3} \qquad O_2 + Fe^{+2}$$

$$2\ O_2^- + 2\ H^+ \qquad H_2O_2 + O_2$$

$$Fe^{+2} + H_2O_2 \qquad OH* + OH^- + Fe^{+3}$$
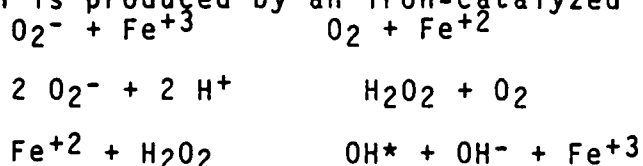
This reaction is favored by iron chelators such as EDTA and DETAPAC.[33] The nature of physiological iron chelators which might resemble EDTA are not known. The involvement of the hydroxyl radical in physiological lipid peroxidation systems remains unclear.[4, 72, 73]

The second in vitro mechanism for the initiation of lipid peroxidation involves nucleotide-iron chelates. In the presence of ADP-Fe^{3+}, superoxide causes rapid lipid peroxidation.[74] Moreover, ADP-Fe^{2+} itself seems to undergo an autoxidation reaction in the presence of O_2 to produce the ADP-perferryl ion (ADP-Fe^{3+}-O_2^-) which can directly initiate lipid peroxidation.[4] Since OH* traps fail to inhibit either of these peroxidation reactions, the OH* radical does not seem to be essential for initiation of peroxidation.[4, 62, 72, 74]

Intracellular iron stores are normally closely controlled by incorporation of the metal into the proteins ferritin and transferrin. If catalytically available intracellular iron were shown to rise significantly during ischemia or reperfusion, this would make available inside the cell during reperfusion the elements to initiate both pathways of lipid peroxidation outlined above.

Once lipid peroxidation is initiated, the reactions can continue via the established propagation reactions:

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1. Lipid alkyl radical + O_2 lipid peroxy radical
    (L·)   (LOO·)
2. Lipid peroxy radical + fatty acid   lipid
    hydroperoxide + lipid alkyl radical
    (LOO· + LH   LOOH + L·)

The lipid peroxy radical is further degraded by oxidation
reactions, giving rise to many products such as MDA, ethane,
pentane, etc.   Termination reactions are possible with
scavengers such as sulfhydryl containing amino acids and vitamin
E.

CEREBRAL ANOXIA, IRON, AND RADICAL INJURY, A SYNTHESIS OF DATA:

The most confusing and controversial data emerging in the
last 20 years from the intense study of CNS ischemia and anoxia
has been the difference in injury produced by complete ischemic-
anoxia versus severe incomplete ischemia or anoxia alone.   This
data pool can be summarized as follows:

1. Severe incomplete ischemia (CNS perfusion rates of 5-
    10% normal) produces greater functional neurologic
    injury than similar periods of complete ischemic-
    anoxia.[31, 75, 76]
2. Severe incomplete ischemia results in worse
    mitochondrial injury than complete ischemic-
    anoxia.[77]
3. Severe incomplete ischemia produces more structural
    derangement during the insult than complete ischemic
    anoxia.[78]
4. Hyperglycemia during ischemia exacerbates injury.[39]

These data have been attributed to a more severe lactic acidosis
with incomplete ischemic or primary anoxia.   However, attempts
to ameliorate the injury with the aggressive correction of
acidosis with agents which will cross the blood brain barrier
have been disappointing.   Nor is any injury produced with severe
hypercapneic brain acidosis.[79]

It was well established that reducing equivalents
accumulate during anoxia or ischemia.[55]   It seems quite likely
that this accumulation of reducing equivalents will be greater,
as is the accumulation of lactic acid,[79] when the CNS is
exposed to a continuing supply of glucose substrate during
simple anoxia or severe incomplete ischemia.   These reducing
equivalents might have been involved in the release of ferrous
iron from ferritin[59] during ischemia, creating a situation in
which tissue injury by radicals was greatly accelerated during
reperfusion.

Alternatively, the metabolism of arachidonic acid by
cycloxygenase and lipoxygenase and xanthine oxidase activity
during severe incomplete ischemia or post-ischemic reperfusion

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would be expected to generate O_2^-. O_2^- acts directly on ferritin

$$Ferritin (Fe^{3+}) + O_2^- \qquad Fe^{2+} + O_2$$
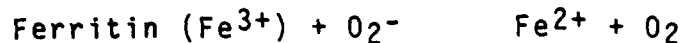
to release soluble ferrous ion.[80]   The pH level may be important to this reaction, since reduced pH accelerates both the reductive release of ferritin bound iron[81] and the rate of invitro brain lipid peroxidation.[82]   Such a mechanism would predict iron release to low molecular weight species (LMWS) only during reperfusion or severe incomplete ischemia.

Based on the above data we predicted that the release of iron would be greater during insults which encompass a period of severe incomplete ischemia, and we predicted that peroxidative brain injury will be ameliorated with deferoxamine.

Deferoxamine is a selective chelator of ferric iron with a dissociation constant for the iron of 10^{-31}M.   Deferoxamine readily crosses the blood brain barrier, and at 5 hours post administration the brain has the highest tissue concentration in the body.[83]   Detectable brain tissue concentrations persist for 24 hours.[84]   The ferrioxamine complex is rapidly excreted in the urine.

Introduction of this chelator into invitro lipid peroxidation systems stops the reactions.[4, 73]   The agent is clinically available for use in acute iron overdose and iron overload syndromes including those related to chronic hemolytic anemias and hemochromatosis.   Ward[85] has recently reported strong evidence for radical mediated lung injury in a model of respiratory distress syndrome produced with complement activation by cobra venom.   Tissue injury in this study was exacerbated by the IV infusion of small quantities of $FeCl_2$, and tissue injury was completely prevented by the administration of deferoxamine.   This study provided the first direct evidence that iron is involved in pathophysiologic injury mediated by radicals and that deferoxamine can be used to directly abort invivo radical mediated critical tissue injury.

Thus, the evidence we have reviewed includes the following:

1.   the persistently unacceptable neurologic morbidity following resuscitation,
2.   the suggestion that major aspects of ischemic brain damage may occur post resuscitation may be in part due to radical injury of tissues,
3.   the evidence indicating that free iron may be increased in the post ischemic brain,
4.   the 'demonstrated role of iron in tissue injury mediated by radicals,
5.   and the fact that in one model system deferoxamine has aborted such injury.

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This evidence made immediate investigation of the status of iron and its association with lipid peroxidation in the post-ischemic brain important. The timing of iron availability was important mechanistically and to characterize a potential therapeutic window. Because lipid peroxidation would be expected to injure membranes, aspects of membrane function (ionic tissue gradients) and structural integrity were important.

OUR APPROACH TO THIS PROBLEM 9/1/84 - 8/31/85

1. Tissue iron and lipid peroxidation following cardiac arrest and resuscitation.

    We first carried out experiments to determine if the species with which tissue iron was associated changed during ischemia or reperfusion. We did this by characterizing the molecular weight of these species in tissue homogenates using ultrafiltration techniques. Fifteen minute cardiac arrest and resuscitation by internal massage in dogs was the animal model we used in these experiments. Rather than go through the details of methodology here in this annual report, we have included the already published paper in the appendices to this report.[5, 6] The methodology is presented in detail in these papers. The results of these studies demonstrated that there was a three fold increase in iron associated with species weighing less than 30,000 daltons during the first 2 hours following resuscitation. There is no tissue iron delocalization to association with LMWS during complete ischemic times of up to 45 minutes. Moreover, there is no significant change in total tissue iron content during ischemia or up to 8 hours following resuscitation (4th major experiment this section). Thus, the phenomenon is one of delocalization of the iron to association with LMWS during reperfusion. With respect to the mechanistic hypothesis for iron delocalization suggested above, these findings favor superoxide dependent reduction of iron from its normal storage forms (predominantly ferritin) during reperfusion, as has recently been demonstrated in vitro by Thomas et al.[80] We have isolated dog brain ferritin using protein electrophoresis and immunoassay techniques. We have found no evidence that the absolute tissue content of ferritin is altered, and we are working to characterize the iron content of dog brain ferritin in wt Fe/wt ferritin in normal and post-ischemic dog brain.

    This tissue iron delocalization is associated with increases in tissue concentrations of MDA and conjugated dienes, which are products of lipid peroxidation. Administration of deferoxamine (50 mg/kg IV) in the immediate post-resuscitation period results in reduction of the tissue concentrations of LP products measured to levels which are not significantly different from those found in non-ischemic controls.

2. Preliminary outcome trial using deferoxamine and lidoflazine after cardiac arrest and resuscitation.

The above findings and previous work with lidoflazine (See Background) led us to carry out a preliminary prospective blind outcome trial with post-resuscitation treatment with deferoxamine and lidoflazine. Dogs were anesthetized with ketamine (7 mg/kg IV) and Halothane (1-2% by inhalation with the anesthesia machine driven with compressed room air). Using sterile surgical technique, the dogs were instrumented for continuous monitoring of EKG, arterial blood pressure, and pulmonary arterial pressure. Large IV lines were established by femoral cutdown. The bladder was catherized for continuous urinary drainage. A pre-arrest arterial blood gas was used to exclude any animals with PO_2 80 torr. Cardiac arrest was induced by injection of 0.75 meq/kg KCl through pulmonary artery catheter, and confirmed by EKG and arterial pressure monitors. The cardiac arrest period was timed by stopwatch. After exactly 15 minutes of cardiac arrest, resuscitation was begun with ventilation with 100% O_2 and internal cardiac massage via left lateral thoracotomy. As in our other studies,[1-3, 9, 33, 38] $NaHCO_3$ (8 meq/kg) and epinephrine (20 mcg/kg) were administered at the initiation of resuscitation, and a continuous epinephrine infusion of 5 mcg/kg/minute was maintained during resuscitation. After 5 minutes of cardiac massage, definitive resuscitation was achieved by defibrillation using 1 Joule/kg with internal paddles. Most animals resumed spontaneous circulation after one shock. In the event that resuscitation was not successfully completed on the first shock, the animals were given xylocaine (2 mg/kg IV) and reshocked. All animals resumed spontaneous circulation (ROSC) within 10 minutes of initiation of the resuscitation phase. Immediately following resuscitaion, the epinephrine infusion was stopped, and a low dose dopamine infusion was used as required to support the diastolic arterial pressure at 90 mmHg. The chest was closed in three layers with incorporation of a thoracotomy tube to underwater drainage. Controlled ventilation was maintained with a mechanical ventilator until the animals were able to maintain adequate tidal volume and arterial oxygenation 80 torr with spontaneous breathing on room air. Post resuscitation fluid maintenance was by the HALFD technique.[86] The animals were transferred from the laboratory to the MSU veterinary ICU, and continuous 24 hour intensive care was provided by DVM or MD members of the research team.

The study drugs were randomized by Dr. Curtis Probst, who was not a member of the investigative team. The 36 hours of study drugs or saline placebo solutions were prepared by Dr. Probst and kept in a light tight box until use. The light exclusion was practiced because of slow photodegeneration which occurs in water solutions of deferoxamine. Deferoxamine was administered in an initial dose of 50 mg/kg by IV infusion

over the first 10 minutes post resuscitation. Repeat doses of 25 mg/kg were given at 8, 16, 24, and 32 hours post resuscitation. Lidoflazine was administered in an initial dose of 1 mg/kg by IV infusion over the first 10 minutes post resuscitation. Repeat doses of 0.5 mg/kg were administered at 12 and 24 hours post resuscitation. These doses and times were based on our knowledge of standard clinical dosages and half-life data.[83, 87]

Neurologic deficit scoring was carried out every 4 hours using the protocol detailed in the original grant application. At 36 hours post-resuscitation the animals were sacrificed and immediately subjected to perfusion fixation with gluteraldehyde following a 90 second saline (3 L) washout to remove tissue blood and assure good fixation. H and E stained sections were examined from all areas of the brain detailed in the contract proposal. This histopathologic examination was done by Drs. A. Koestner (Chairman, Pathology, MSU) and Dr. K. Kumar, who are both experienced neuropathologists.

Results of this preliminary study are shown below as mean values and include neurologic deficit scores (NDS), and overall performance category (OPC).

	Treated Animals		Controls	
	NDS%	OPC	NDS%	OPC
4 hours	72%	3.8	69%	3.0
8 hours	69%	3.5	64%	2.7
16 hours	59%	3.2	58%	2.8
24 hours	51%	3.3	50%	2.9
36 hours	50%	2.9	48%	2.7

There were no significant differences in this data by MANOVA analysis using either parametric or non-parametric techniques.

The histopathologic studies were scored by the method of Garcia.[88] Sections from the frontal, parietal, and occipital cortex, and also from hippocampus and cerebellum were studied in each animal. The histopathologic scoring system uses the number of neurons with pyknotic deeply eosinophylic nuclei and the degree of glial reaction (neuronophagia) to establish scores. The histopathologic scoring was not significantly different between the two groups. The general histopathologic pattern noted was characterized by many pyknotic neuronal nuclei, aggressive neuronophagia, and evidence of pseudolaminar necrosis in all animals. The purkinje cell layer of the cerebellar

cortex was most consistently damaged. One interesting and consistent difference was noted between the two groups, which was not reflected in the standard scoring system. Microhemorrhages were prominently present in all areas of the brain in the control animals. No microhemorrhages were found in any of the treated animals.

This finding is interesting in view of the work by Anderson and Means[69] with spinal cord injury in which they showed that introduction of small amounts of $FeCl_2$ into the cord produced an aggressively progressive hemorrhagic lesion with evidence of lipid peroxidation. They were able to quench the injury with radical scavengers.

Our preliminary study does not show any evidence of outcome protection with the drugs and dosages employed. The findings in this preliminary outcome study led us to a series of questions about the pathophysiology and the drugs which we began to address during the last part of this first project year. These include the following:

a. How good is the evidence for tissue calcium overload during the first hours of reperfusion and/or protection from such a phenomenon by a calcium antagonist? We began to address this question with our third major experiment conducted during the first contract year discussed below.

b. Although the literature did not provide a satisfactory answer to #1, it seems clear from studies reviewed in the background section of this report that the calcium antagonists do improve post ischemic perfusion. If tissue injury by oxygen dependent lipid peroxidation turns out to be a major pathologic mechanism during reperfusion, are early interventions to improve tissue reperfusion and oxygen delivery potentially counter-productive? Could it be that the post-ischemic hypoperfusion syndrome in the brain is in part an adaptive defense against oxygen radical injury? Evidence that lipid peroxidation is augmented by early intervention with agents which correct brain hypoperfusion would support this hypothesis. This may emerge from the drug screening experiments we are currently conducting and which we will discuss last in this report.

c. Indeed, could the primary messenger for the post-ischemic perfusion reduction be a product or products of lipid peroxidation? Safar (personal communication) has conducted brain perfusion studies as part of an evaluation of deferoxamine treatment post cardiac arrest and resuscitation. He has found significant

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protection of tissue perfusion by this drug, which we have shown to inhibit brain lipid peroxidation during reperfusion.

d. Is the dose of deferoxamine which we have used adequate? At first glance, the answer would be yes. The dose is within the standard clinical range used for iron overload diseases, and it appears to significantly inhibit lipid peroxidation during the first 2 hours post resuscitation. However, our iron data shows that the brain levels of chelatable iron after cardiac arrest and resuscitation are much greater than those seen in normal physiologic states. Indeed, the levels of LMWS iron are most certainly much higher in the post-resuscitation phase than in even iron overload diseases. If one assumes that the phenomenon of iron delocalization during post-ischemic reperfusion is not limited to the brain, our dose may be significantly too low. This assumption is not unreasonable given the involvement of iron is myocardial reperfusion,(Holt et al., unpublished data) radical mediated lung injury,[85] and post-ischemic radical injury of the bowel.[52] Moreover, while the brain has significant iron content, many other organs contain more. If one generalizes our brain iron delocalization data to the whole body (about 200 mcM/kg), the required dose of deferoxamine for stochiometric binding would approach 150 mg/kg of the drug during the first 2 hours post-resuscitation. One first check of this hypothesis is to examine the general circulation for products of lipid peroxidation. We have preliminary data for arterial thiobarbituric acid reactive substances (TBARS = MDA ?) which indicates that these products are quadrupled after 2 hours of reperfusion in comparison to non-ischemic controls. This would suggest much wider post-resuscitation organ involvement in lipid peroxidation than just the brain. Since the iron containing ferrioxamine complex is excreted unchanged in the urine, it should be possible to do a dose response of total urinary ferrioxamine complex as a function of the amount of drug administered. We plan to do this experiment.

3. Time course of tissue ionic content during reperfusion following a 15 minute cardiac arrest in dogs.

Lipid peroxidation would be expected to adversely effect membrane functions including maintenance of normal tissue ionic concentrations. If the membrane was actually "holed" changes in concentrations of several small ion not usually tightly bound to larger moities would be expected.

Membrane function associated with ionic control is of importance to ultrastructural integrity and cellular survival. Moreover, control of ionic concentrations is vital in excitable tissue for generation of and response to action potential stimuli. Although alterations in brain tissue concentrations of Ca, Na, and K have been noted following various ischemic insults,[11, 12] these changes have not been systematically characterized, nor any attempt made to connect these changes with specific pathochemical or ultrastructural changes. Therefore, this study was undertaken to characterize the time course of brain tissue content of Ca, Mg, Fe, Na, and K during reperfusion after a 15 minute cardiac arrest in dogs.

Animal preparation and groups:

Twenty five large mongrel dogs weighing between 22 and 28 kg were utilized in the study after heart worm screens by techniques for both microfilaria and immuno-flourescence were negative. The animals were anesthetized with ketamine (7 mg/kg IV) and deep surgical anesthesia was established and maintained with 1-2% halothane. All animals were intubated and placed on controlled ventilation using a Bird ventilator and compressed room air. A femoral artery was catherized, and the catheter was connected to a transducer and pressure monitor for continuous display of the arterial blood pressure. A catheter was introduced into the pulmonary artery via a right jugular vein cutdown, with catheter placement being guided by waveform display via connection of the catheter to a transducer and pressure monitor. The bladder was catheterized for continuous drainage. Large bore IV lines were established in one forelimb and in a femoral vein via cutdown. Pre-arrest maintenance IV fluids were given with isotonic saline. Continuous EKG rhythm monitoring was done using a Life Pac II monitor equipped with internal defibrillator paddles.

The dogs were divided into five experimental groups of five animals each.

1. Non-ischemic controls.
2. Ten minutes reperfusion. Fifteen minute cardiac arrest was induced by injection of 0.75 meq/kg KCl through the pulmonary arterial line. Cardiac arrest was confirmed by arterial pressure and EKG monitors. The 15-minute cardiac arrest was followed by 10 minutes of internal cardiac massage (ICM) via left lateral thoracotomy without restoration of spontaneous circulation (ROSC). Ventilation with 100% oxygen was begun at the initiation of ICM, and $NaHCO_3$, 8 meq/kg and epinephrine, 20 mcg/kg, were administered at the beginning of ICM. Epinephrine infusion at 5 mcg/kg/min was maintained throughout the 10 minutes of ICM, using a Harvard infusion pump.

3. Fifteen minute cardiac arrest followed by 2 hours ofintensive care. The resuscitation protocol was the same as that in group 2, except that ROSC was established by defibrillation at 30 Joules using internal paddles after 5 minutes of ICM.
4. Fifteen minute cardiac arrest followed by resuscitation and 4 hours of post-resuscitation care.
5. Fifteen minute cardiac arrest followed by resuscitation and 8 hours of post-resuscitation care.

Pre-arrest blood gasses were done in all animals. PO_2 ranged between 80 and 100 in all animals pre-arrest.

The post-resuscitation period before brain tissue sampling was timed from the initiation of ICM in all animals. Post-resuscitation care utilized the HALFD fluid regimen[86] to support PA diastolic pressures at between 10 and 20 mM Hg. Arterial diastolic pressure was maintained at 80 to 90 mM Hg with dopamine infusion when required. Periodic blood gas determinations were used to guide ventilation so as to maintain PCO_2 between 35 and 45 mM Hg, PO_2 80 mM Hg, and pH between 7.3 and 7.45.

Brain tissue harvesting and analysis:

Shortly before the indicated time for brain harvesting, the scalp was surgically reflected and the parietal skull opened using a 15 mm surgical trephine. At the time of tissue harvesting, cardiac arrest was induced by injection of .75 meq/kg KCl through the PA line, and a 2 to 3 gm sample of the pareital cortex was immediately taken.

The tissue sample was immediately placed in ice cold Ringer lactate and exactly 1 gm of tissue was weighed out using a Metler balance. The 1 gm of tissue was placed in a 50 mL volumetric flask, and 10 mL of absolute nitric acid was added. This was gently heated until the tissue was fully dissolved and the solution was clear. The solution was capped and allowed to cool overnight. The solution was then diluted with triple distilled water to approximately 25 mL, and 500 uL of a Ytrium standard for the atomic emission spectrometer was added. The solution was then diluted to exactly 50 ml. The content of Ca, Mg, Fe, Na, and K was determined in triplicate using a Jarrel Ashe inductively coupled plasma atomic emission spectrometer with a detection limit of 10^{-9}. The mean value of the three analytical determinations was reported as mceq/gm tissue wet weight.

All data for the five dependent variables (Ca, Mg, Fe, Na, and K) across the five sampling times was examined by the analytical method of multivariance using the SPSS program on Michigan State University's Cyber 170 mainframe computer. This

omnibus test for significance in the data pool was followed by univariate analysis of variance for each of the five dependent variables. When significant differences were found within the dependent variables, they were pinpointed using Scheffe post-hoc analysis.

Results of this study are shown in Figures IV and V. Omnibus statistical analysis of the data by MANOVA gave p .001 for significant differences in the study. Univariate analysis of variance for Ca yielded a $F = 5.22$; p .005: for Na, $F = 8.69$; p .001: for K, $F = 5.21$; p .005. Individually significant differences within the groups at alpha = .05 are as follows:

a. There are small but significant increases in both Na and Ca tissue content after 10 minutes of reperfusion by ICM.

b. All five ions are indistinguishable from non-ischemic controls after 2 and 4 hours of reperfusion.

c. After 8 hours of reperfusion, mean values for Na and Ca content have doubled, and K content is halved. These data are all significant. There are no significant changes in total tissue iron (Mean all studies = 310 mcM) or magnesium (Mean all studies = 4.81 mM) content.

The failure of Mg or Fe concentrations to change argues against significant dilutional changes occurring in the tissue by edema. The large shifts in Ca, Na, and K, in the direction of equilibration with plasma and extracellular fluid concentrations, argue that large numbers of cells in the sampled tissue have become unable to maintain normal transmembrane gradients of these ions after between 4 and 8 hours of reperfusion. At least two potential mechanisms could explain this. Progression of membrane injury by lipid peroxidation to the point of physical holes being present in the membrane would be consistent with failure of ionic homeostasis. Alternatively, profound ATP depletion could also result in equilibration of ionic species down their membrane concentration gradients. Indeed, both mechanisms could operate jointly.

Recognition of 8 hours post resuscitation as a critical break point in membrane functional integrity in this model of cardiac arrest and resuscitation was very important to us. This experiment suggests that the time window for therapeutic intervention may extend at least 4 hours into the reperfusion phase. Moreover, we have found no evidence of net changes in tissue Ca concentrations which are not associated with major shifts in other ions. This suggests that the pathochemical processes are more general that can be readily attributed to changes in specific Ca conductance. We now predict that specific Ca channel blockers may have little effect on tissue Ca

content during the reperfusion phase. We are currently studying four different Ca antagonists in our drug screening study, and should have this data shortly. Finally, identification of a major membrane functional break point after 8 hours of reperfusion has given us a specific time to further characterize pathochemistry and ultrastructural injury, and also to test reputed therpeutic interventions against. We are also examining tissue concentrations of products of lipid peroxidation, saturated to unsaturated fatty acid ratios, and the LMWS content of iron after 8 hours reperfusion.

4. Ultrastructural changes after 8 hours of reperfusion following a 15 minute cardiac arrest and resuscitation.

We have now used the cardiac arrest and resuscitation model above to characterize ultrastructural changes in brain tissue from the cortex, cerebellum, and hipocampus in five non-ischemic controls and five animals after 15-minute cardiac arrest and 8 hours of reperfusion. At the appropriate time the animals are anesthetized, and the chest entered through the left lateral thoracotomy. The pericardium is widely opened. Clamped large bore bypass catheters are placed in the left and right ventricles and sutured tightly in place with a 1 chromic pursestring. The animal remains alive with good spontaneous perfusion during this brief procedure. A cardiac bypass pump is then used to flush the animal with 3 liters of normal saline at a perfusion rate of 2.5 L/min. The aorta is crossclamped just below the left subclavin immediately before the bypass pump is started. After the 70 second saline washout, the animal is perfusion fixed with 7 liters of the Karnovsky solution modified for calcium precipitation by the method of Borgers et al.[89] This is also run at 2.5 L/minute. The animal is then immediately autopsied, and the already fixed brain placed in the fixative solution overnight. The brain is then sectioned and stained by the method of Van Reempts et al.[13] for ultrastructural study and localization of cellular calcium. The findings of this study in conjunction with the ionic studies have been submitted to the International Academy of Pathologists for presentation at their annual meeting.

The numerous electron photomicrographs have been evaluated both by Drs. Kumar and Koestner here, and also by Dr. Marcel Borgers in Beerse, Belgium. The following ultrastructural alterations are consistently seen after 8 hours of reperfusion, and are not present in non-ischemic controls.

a. Dendritic end plates are empty of vesicles and have some fine deposition of calcium precipitates.
b. Nuclear chromatin is densely clumped.
c. Nuclear and plasma membranes have obviously deteriorated structural definition, and numerous holes in the continuity of these membranes are evident.

d. The concentration of lysozymes is greatly increased. These lysozymes are almost invariably intimately associated with lipid micelles which do not appear to be membrane bound. The micelles may represent hydrophobic fatty acid debris which has been removed from normal membrane structures by the chemical reactions suggested above.

e. Evidence of intracellular edema is present.

Interestingly, mitochondrial structure is generally well preserved, and heavy mitochondrial calcium precipitates are not frequently seen. Moreover, there is no evidence yet of glial edema or proliferation.

These findings, coupled with direct evidence for lipid peroxidation during early reperfusion and collapse of tissue ionic gradients only after more than 4 hours of reperfusion present a coherent picture. This emerging picture is consistent with a major role for lipid peroxidation during reperfusion in the pathochemistry and structural damage seen in the brain after cardiac arrest.

5. <u>Brain tissue iron delocalization during various artificial perfusion methods following a 15 minute cardiac arrest.</u>

This study has been submitted for publication.[7] Therefore, details of the methods will not be presented here. Briefly, five groups of five animals each were studied for brain tissue content of LMWS and MDA. The experiment modeled potential choices of artificial perfusion techniques during attempted resuscitation from a 15 minute cardiac arrest. The experimental groups were:

a. Non-ischemic controls.

b. 45 minute cardiac arrest without resuscitation.

c. 15 minute cardiac arrest followed by 30 minutes of conventional CPR.

d. 15 minute cardiac arrest followed by 30 minutes of interposed abdominal compression CPR (IAC-CPR)[90]

e. 15 minute cardiac arrest followed by 30 minutes of ICM.

Initial epinephrine (20 mcg/kg) was administered to all three resuscitation groups and followed by continuous epinephrine infusion. Initial doses of $NaHCO_3$ were chosen based on our previous experience with these resuscitation techniques, and with the object of generating similar pH between the groups after 10 minutes of artificial perfusion.

We were unable to eliminate significant differences between the resuscitation models in PCO_2, PO_2, and HCO_3^- during the resuscitations. Hyperoxia and hypocarbia were consistently

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noted in the CPR and IAC-CPR models, together with progressive significant falls in HCO_3^-. In contrast, PCO_2, pH, and HCO_3^- varied little during the 30 minute resuscitation with ICM. We attribute these findings to the well established low cardiac outputs seen with the closed chest methods. The persistent hypocarbia and hyperoxia in the closed chest methods is attributed to an increased ventilation/perfusion ratio in the lungs. This reflects the low cardiac outputs with closed chest resuscitation techniques, which would result in extended exposure of pulmonary capillary blood to gas exchange at the alveoli.

We suggest these blood gas patterns reflect inherent characteristics of the different massage techniques, and thereby are not improper variability in this study. Given this, there are significant differences in the brain tissue content of LMWS iron. That associated with CPR is more than double those associated with non-ischemic controls, 45 minute arrest without intervention, and ICM. There is also a 75% increase in tissue LMWS iron seen with IAC-CPR, although this did not achieve statistical significance in this study.

We conclude that ICM is a superior resuscitation technique following prolonged cardiac arrest for both stability of blood gas parameters and for its low initial rate of iron delocalization. These findings tend to support our hypothesis that the rate of brain iron delocalization into LMWS would be greater during insults encompassing a period of severe incomplete ischemia. These findings are also interesting in light of our previous demonstration of major injury to brain mitochondrial state 3 respiration[91] during closed chest massage techniques. The demonstration by Ernster and Hillered[61] that exposure of brain mitochondria to an iron catalysed oxygen radical system produces deep inhibition of mitochondrial state 3 respiration is also interesting in this regard. Taken together, these data suggest that iron delocalization during severe incomplete ischemia produced by inadequate artificial perfusion techniques may result in profound mitochondrial injury during the resuscitation phase, which may not be expected to recover readily following ROSC. Outcome studies by Safar's group[16] demonstrate consistently brain dead animals after 30 minutes of closed chest massage techniques following only a 4 minute cardiac arrest. In contrast, animals resuscitated by ICM were neurologically normal. Our data, the studies by Safar, and the clinical outcome data covered in the background section of this report, all suggest that early resort to ICM may be appropriate to protect the brain during resuscitation attempts when the initial treatment has failed to rapidly establish ROSC.

CONCLUSIONS

Our first year contract studies demonstrate a massive phenomenon of iron delocalization to LMWS during reperfusion following prolonged cardiac arrest. This occurs during the first 2 hours of reperfusion, and is accompanied by evidence of lipid peroxidation in the brain. The products of lipid peroxidation are returned to normal levels by treatment in the post-resuscitation phase with deferoxamine.

Tissue recovery of normal ionic content of Na, Ca, and K is rapid during reperfusion. Following a 15 minute cardiac arrest and resuscitation, normal tissue content of these ions is maintained for at least 4 hours. However, between 4 and 8 hours of reperfusion, control of tissue ionic content is lost with a massive trend toward equilibration of the total tissue ionic content with that of plasma. This phenomena is not limited to calcium shifts, and may be expected to be unlikely to respond favorably to administration of calcium antagonists during reperfusion. This phenomena is associated with ultrastructural evidence of holes in plasma and nuclear membranes, clumping of nuclear chromatin, empty dendritic endplates, and surprisingly little mitochondrial structural injury.

An initial outcome study using a calcium antagonist and deferoxamine together failed to demonstrate any significant improvement in neurological outcome in comparison to good intensive care alone. Moreover, classic histopathologic changes were not affected between the two groups at 36 hours post resuscitation. However, the absence of microhemorrhages in the treatment group was a significant difference and is encouraging. Subsequent stochiometric calculations of likely dose requirements for deferoxamine suggest that the dose was inadequate in this study. Moreover, the ionic and ultrastructural studies do not intuitively support a major role for calcium antagonists in amelioration of the reperfusion injury.

The study of the different artificial perfusion methods indicates, as predicted by our chemical hypothesis, that tissue iron delocalization is accelerated by resuscitation techniques which do not produce adequate brain perfusion.

PLANS AND RECOMMENDATIONS

We now have an established sequence of pathochemical and ultrastructural injury which occurs during reperfusion of the brain following a 15 minute cardiac arrest. We are thus in an ideal position to rapidly screen a number of potential therapies aimed at the injury mechanisms described above to see if they

a. Inhibit iron delocalization
b. Inhibit lipid peroxidation
c. Inhibit collapse of tissue ionic content seen at 8 hours of reperfusion
d. Inhibit ultrastructural injury seen 8 eight hours of reperfusion.

We are examining the following agents across these screens: SOD, inhibitors of lipoxygenase, cycloxygenase, and xanthine oxidase, catalase, iron chelators, mannitol, and a variety of calcium antagonists.

Agents which demonstrate effectiveness against the above systematic screening studies will be studied in the long term outcome trials detailed in our contract proposal.

Our first year budget was adequate, although we needed to make some category shifts (from ICU care to supplies) which I wrote you about.

We would like to request that 15% salary support ($7,365.00 salary, + $1,731.00 (fringe = 23.5%), = $3,729 (41% indirect costs) = $12,825.00 total) be added to the second and third years of the contract for Dr. Kusam Kumar. Dr. Kumar has worked enthusiastically with us in characterizing the histopathology of global brain ischemia by both light and electron microscopic techniques. In this she has had the solid support and assistance of her Departmental Chairman, Dr. A. Koestner, whose special field is neuropathology. In studying the literature, it is surprising that there have been minimal systematic histopathological studies of global brain ischemic injury. The only investigator we have found committed to this in the United States is Dr. Garcia at the University of Alabama. Dr. Kumar's help has been invaluable during the first year as we have further sorted out the reperfusion brain injury problem. We are delighted to have her on our team, and it would be most helpful to be able to give her at least some minimal salary support.

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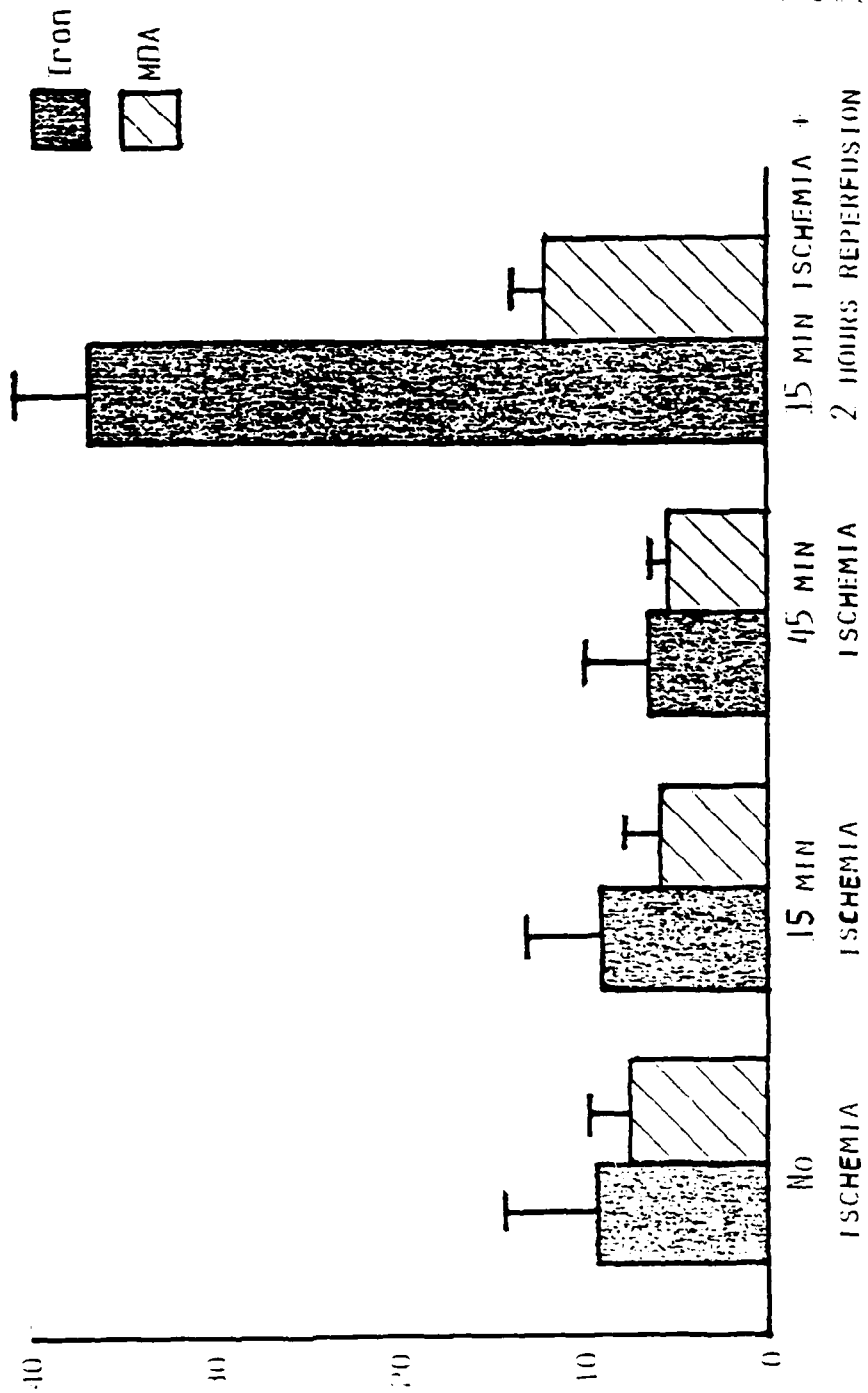
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Figure I: Tissue ... ...
Iron and Mal... after
Cardiac Arrest and Resuscitation

Iron

MDA

NO ISCHEMIA

15 MIN ISCHEMIA

45 MIN ISCHEMIA

15 MIN ISCHEMIA + 2 HOURS REPERFUSION

40

30

20

10

0

FIGURE II

BRAIN TISSUE LMWS IRON AND LIPID PEROXIDATION PRODUCTS
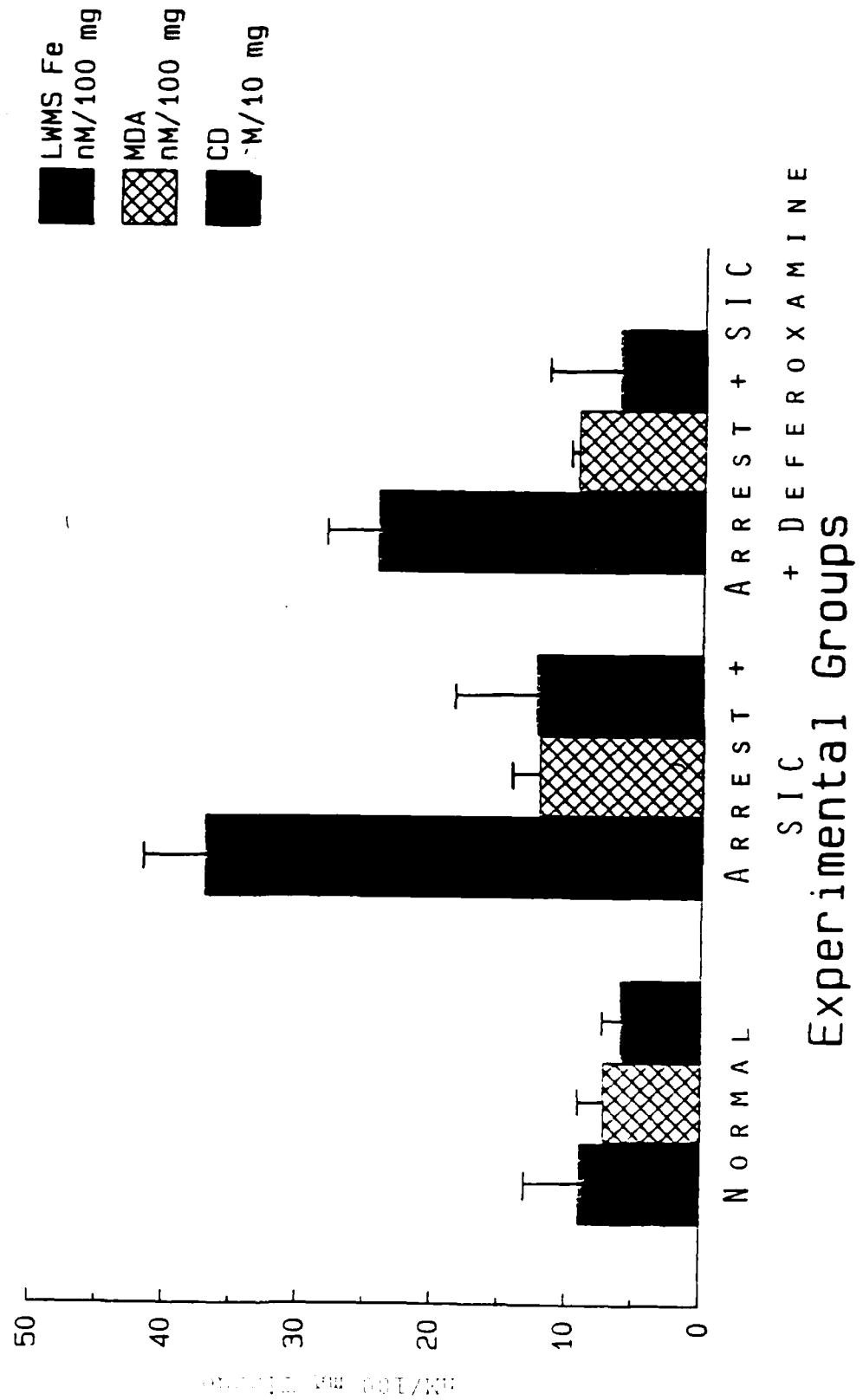
AFTER 15 MIN CARDIAC ARREST AND 2 HOURS REPERFUSION

LWMS Fe
nM/100 mg

MDA
nM/100 mg

CD
nM/10 mg

50 — 40 — 30 — 20 — 10 — 0

nM/100 mg TISSUE

NORMAL   ARREST +   ARREST +   ARREST + SIC
          SIC   + DEFEROXAMINE

Experimental Groups

FIGURE III: Brain tissue LMWS Iron after 15 minute cardiac arrest and various reperfusion methods.

Ultrafiltered Iron
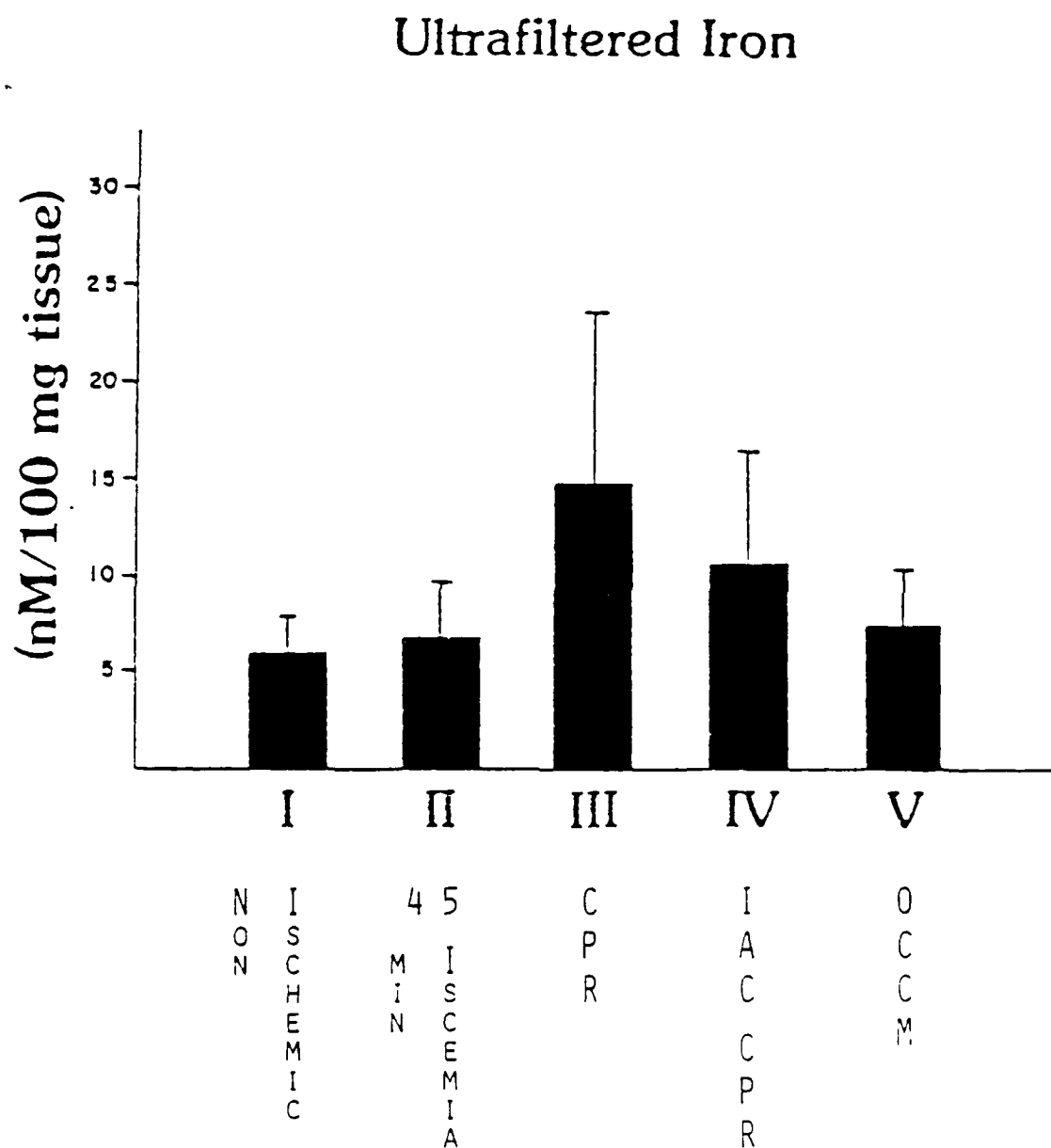
Figure IV

Time Course of Brain Cortex Calcium
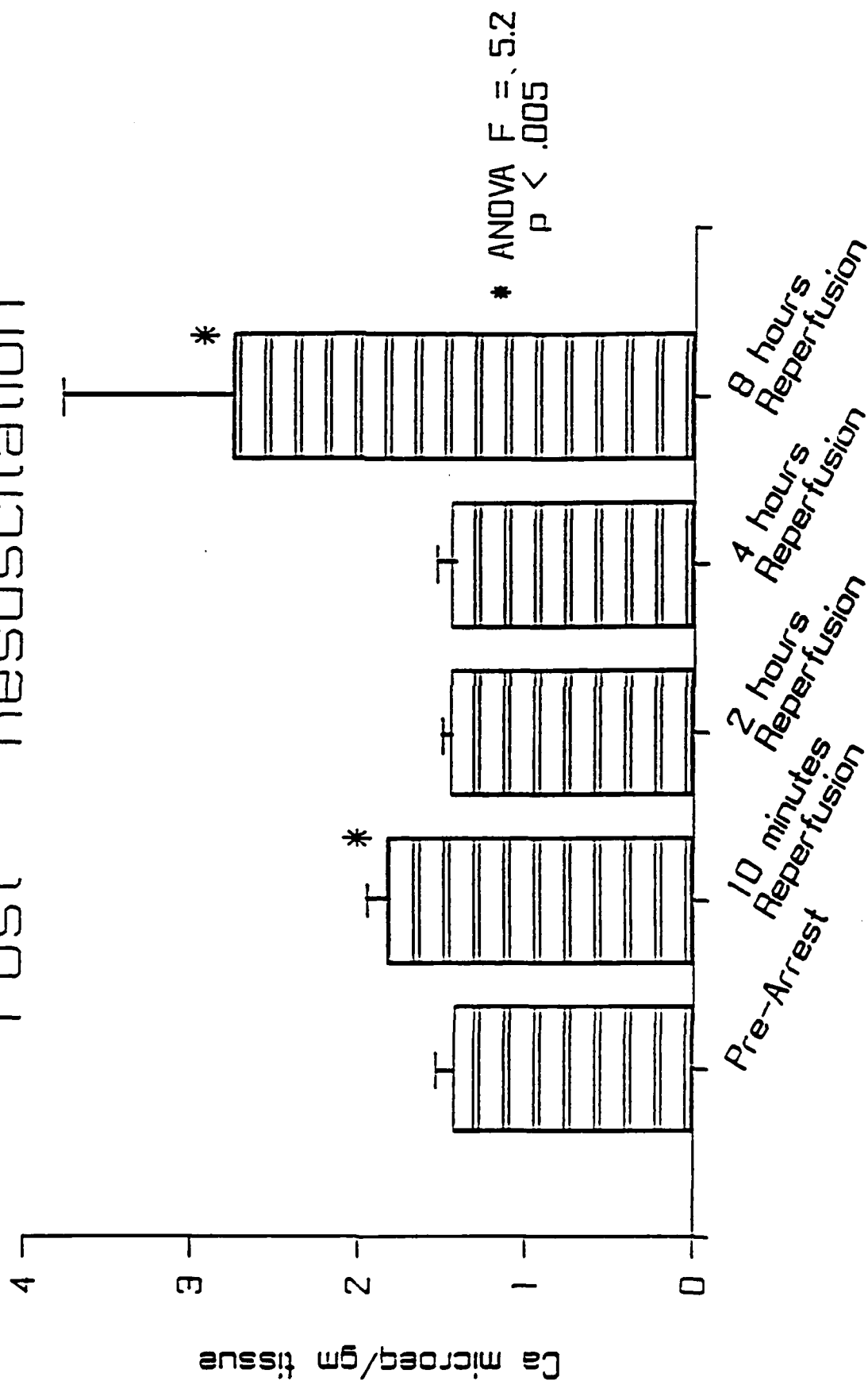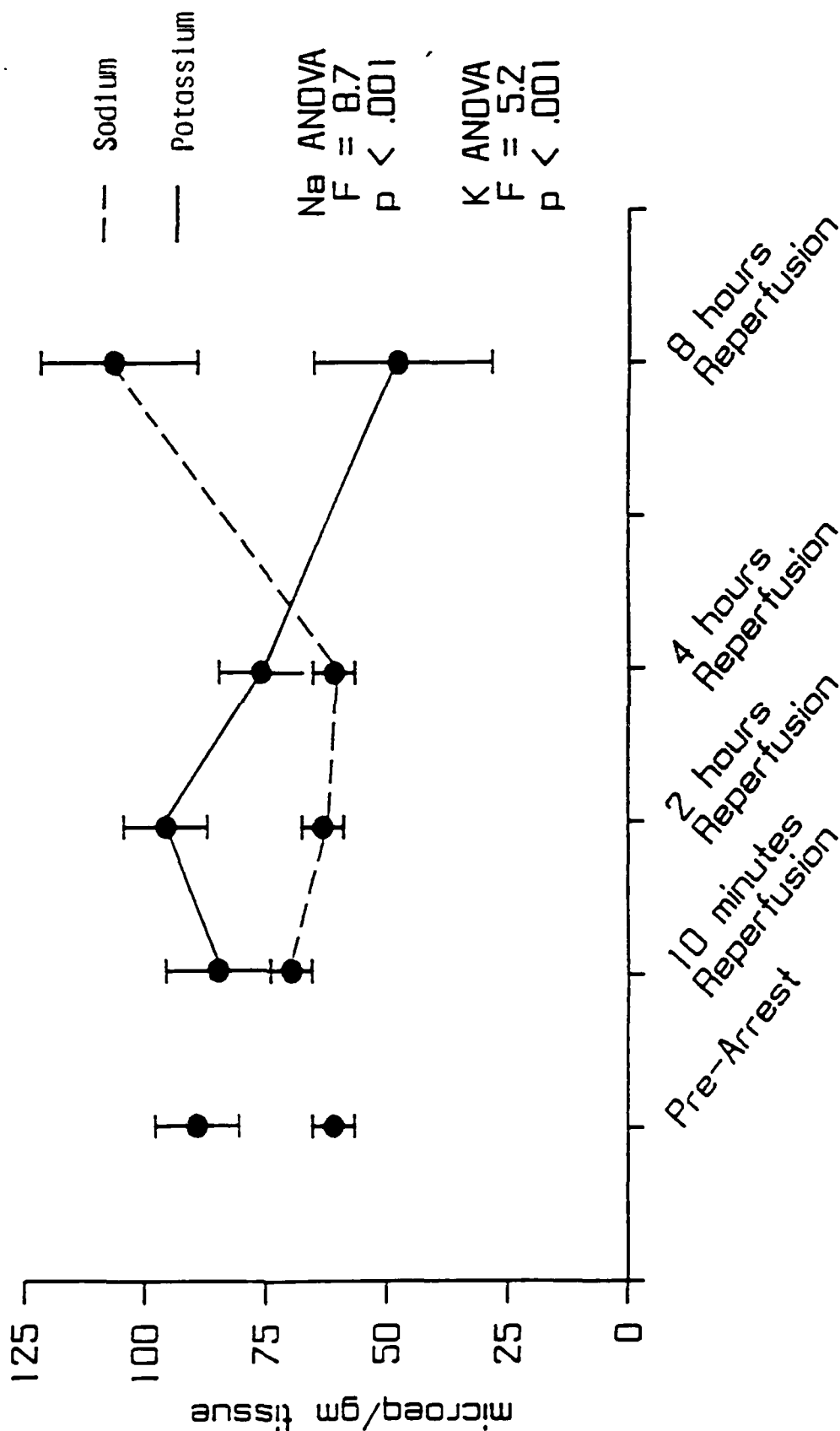## Post - Resuscitation

Ca microeq/gm tissue

ANOVA $F = 5.2$
$p < .005$

Figure V

Time Course of Brain Cortex
Sodium and Potassium
Post-Resuscitation

- - - Sodium
—— Potassium

Na ANOVA
F = 8.7
p < .001

K ANOVA
F = 5.2
p < .001

Pre-Arrest

10 minutes
Reperfusion

2 hours
Reperfusion

4 hours
Reperfusion

8 hours
Reperfusion

microeq/gm tissue

40

END

DATE

FILMED

6-1988

DTIC